Title

Modeling human trophoblast, the placental epithelium at the maternal fetal interface.

Journal

Reproduction (Cambridge, England), 160(1)

ISSN

1470-1626

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Publication Date

2020-07-01

DOI

10.1530/rep-19-0428

Peer reviewed

Title: **Modeling human trophoblast, the placental epithelium at the maternal fetal interface**

Running title*:* Modeling human placental epithelium

Summary sentence:
This review focuses on model systems of human trophoblast differentiation, including advantages and limitations of stem cell-based culture, trophoblast organoid, and organ-on-a-chip methods and their applications in understanding placental development and disease.

Keywords*:* Placenta, Cytotrophoblast, Extravillous trophoblast, Syncytiotrophoblast, Pluripotent stem cells, trophoblast stem cells, trophoblast organoid, placenta-on-a-chip.

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Grant support:
This work was supported by funds from NIH/NICHD (R01-HD089537 to M.M.P. and K99-HD091452 to M.H.). O.T. was supported by the Bridges to Stem Cell Research Internship Program Grant from the California Institute for Regenerative Medicine (CIRM EDUC2-08376).

Conference presentation:
This work was partially presented by Dr. Parast at the Gordon Research Conference on Mammalian Reproduction in July 2018, in Lucca, Italy.

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Abstract

Appropriate human trophoblast lineage specification and differentiation is crucial for the establishment of normal placentation and maintenance of pregnancy. However, due to the lack of proper modeling systems, the molecular mechanisms of these processes are still largely unknown. Much of the early studies in this area have been based on animal models and tumor-derived trophoblast cell lines, both of which are suboptimal for modeling this unique human organ. Recent advances in regenerative and stem cell biology methods have led to development of novel *in vitro* model systems for studying human trophoblast. These include derivation of human embryonic and induced pluripotent stem cells and establishment of methods for the differentiation of these cells into trophoblast, as well as the more recent derivation of human trophoblast stem cells. In addition, advances in culture conditions, from traditional two-dimensional monolayer culture to three-dimensional culturing systems, have led to development of trophoblast organoid and placenta-on-a-chip model, enabling us to study human trophoblast function in context of more physiologically accurate environment. In this review, we will discuss these various model systems, with a focus on human trophoblast, and their ability to help elucidate the key mechanisms underlying placental development and function.

Introduction

Trophoblast (derived from the Greek word "tropho," meaning to feed) is the epithelial cell in the placenta, a transient organ which plays a pivotal role in fetal growth and development during pregnancy. Understanding trophoblast differentiation is crucial for unraveling placental development and function across gestation, as well as placenta-based complications of pregnancy. Abnormal trophoblast differentiation has been associated with numerous pregnancy complications, including recurrent miscarriage, preeclampsia, and fetal growth restriction (Jauniaux et al. 2006; Norwitz ER 2006; Romero et al. 2011).

Placental epithelium is derived from the trophectoderm—the outer layer of the pre-implantation blastocyst-stage embryo, which gives rise to three distinct trophoblast subtypes. Cytotrophoblast (CTB) are considered proliferative stem/progenitor cells. Within floating villi, CTBs undergo cell-cell fusion to form a multinucleated syncytiotrophoblast (STB), which secretes human chorionic gonadotropin (hCG), placental lactogen (hPL, also called chorionic somatomammotropin hormone or CSH) and pregnancy-specific glycoproteins (PSGs), and is involved in nutrient/gas exchange. Within trophoblast cell columns of anchoring villi, CTBs differentiate into extravillous trophoblast (EVT), a highly motile cell type which invades the maternal uterine wall to remodel spiral arteries and establish blood supply to the feto-placental unit (Bischof and Finger 2005; James et al. 2012).

As the most evolutionarily divergent organ, the human placenta, and specifically human trophoblast, have been difficult to study, mostly due to the lack of reproducible and widely-available model systems which accurately represent these cells *in vivo*. However, a combination of recent advances in regenerative medicine has greatly expanded our ability to model this important cell type at the maternal-fetal interface. These include the ability to reprogram a wide range of somatic cell types into induced pluripotent stem cells (iPSCs) (Takahashi et al. 2007), which can subsequently be differentiated into trophoblast (Amita et al. 2013; Horii et al. 2016), and establishment of culture conditions for growth and expansion of both human trophoblast stem cells (hTSC) (Okae et al. 2018) and self-replicating human placental organoids (Haider et al. 2018; Turco et al. 2018). Most recently, several groups have also brought together various cell types to develop placenta-on-a-chip models (Blundell et al. 2016; Arumugasaamy et al. 2018; Nishiguchi et al. 2019). In this review, we will describe these recent advances, detailing the advantages and limitations of each model system, and placing them in the larger context of both the past and future of this important area of reproduction.

Human trophoblast lineage specification and early placental development

Much of what we know about TE establishment comes from studies in mice, which have identified a primary role for the Hippo signaling pathway in this process (Nishioka et al. 2009). Specifically, this pathway is turned off in the outer (TE) cells of late-stage morula, leading to formation of a Tead4-Yap1 complex, which subsequently induces Cdx2, a master switch of TE cell fate (Strumpf et al. 2005). Downstream of Cdx2, other transcription factors are induced, including Eomes and Elf5, which help to maintain proliferative mouse trophoblast stem cells (mTSC) within the extraembryonic ectoderm layer of the early post-implantation embryo (Russ et al. 2000; Donnison et al. 2005; Senner and Hemberger, 2010). Gata2 and Gata3 transcription factors have also been found to play significant, albeit redundant, roles in establishment of TE (Home et al. 2017).

Little is known about the mechanism(s) behind specification of trophectoderm (TE) in the human embryo. Recent studies, using a combination of immunostaining and single-cell

RNAseq, have identified significant differences in marker expression between mouse and human pre-implantation embryos, including absence of ELF5 and EOMES in human TE (Niakan and Eggan, 2013; Yan et al. 2013; Blakeley et al. 2015; Petropoulos et al. 2016). In addition, several lines of evidence, including a prolonged period during which the pluripotency marker POU5F1/OCT4 and the TE marker CDX2 are co-expressed in the TE layer, suggest that human TE is not specified until at least the late blastocyst stage (Niakan et al. 2012; Niakan and Eggan 2013). In fact, TE cells, taken from a day 5 human blastocyst and repositioned into the center of the same embryo, did not sort back to their original position and induced expression of NANOG, an epiblast marker (De Paepe et al. 2013). Furthermore, while Pou5F1/Oct4 is not involved in TE specification in mouse, the targeted knockout of its ortholog in the human blastocyst was found to affect gene expression in both the epiblast and TE compartments (Fogarty et al. 2017). Finally, methods have been developed allowing human embryos to transition from pre- to post-implantation stages *in vitro* (Deglincerti et al. 2016; Shahbazi et al. 2016); these studies have shown GATA3 to be the most uniform marker of human TE, with CDX2 expressed less consistently among TE cells. While all the above data point to numerous differences in mechanism(s) of TE fate specification in the human embryo, the exact sequence of events, including the transcriptional networks and signaling pathways involved, remain obscure.

Even less is known about events surrounding implantation of the human embryo, which begins at ~7 days post-fertilization. Studies by Hertig and Rock led to the first observations of this crucial period of development (Hertig et al. 1954; Hertig et al. 1956); however, most other knowledge of these events has arisen from *in vitro* culture of human embryos, performed, in large part, in context of fertility treatment. Studies using human endometrial epithelial monolayers have shown that, in contrast to mouse embryos, the initial attachment of human embryos occurs via the polar TE, cells located adjacent to the inner cell mass (Lindenberg et al. 1989). Subsequently, the embryo appears to dive underneath the endometrium, where, surrounded by endometrial stroma, the TE layer begins to expand and differentiate, giving rise to an inner layer of mononuclear cytotrophoblast (CTB) and an outer layer of primitive syncytium (Hertig et al. 1956; Boyd and Hamilton, 1970). The latter cells are unusual in that, while multinucleated, they appear to be highly invasive, located at the forefront of the burrowing embryo; these cells should be distinguished from definitive syncytiotrophoblast, cells which arise slightly later in gestation, through cell-cell fusion of CTB.

Within a few days following implantation, primary villi begin to form through invagination of the CTB layer; these are subsequently invaded by mesenchymal cells and eventually by fetal blood vessels, and undergo branching morphogenesis, leading to formation of mature chorionic villi, the functional units of the human placenta (Benirschke et al. 2012). Within the placental disc, floating chorionic villi, lined by an outer layer of multinucleated syncytiotrophoblast (STB), serve as the exchange interface for gases and nutrients. At the basal plate (the maternal surface of the placental disc), anchoring villi attach to the uterine wall through trophoblast cell columns, structures composed of a progression of CTB differentiating into extravillous trophoblast (EVT) (Benirschke et al. 2012). Mature EVT are highly motile cells, which invade through the decidualized uterine stroma and up to the inner one-third of the myometrium, remodeling maternal spiral arterioles in order to optimally supply the fetoplacental unit with maternal blood (Benirschke et al. 2012).

Traditional models for study of human trophoblast differentiation

Placental explants can be generated from placental tissues of any gestation. Although numerous culture conditions and uses have been described (reviewed by Miller et al. 2005), generally speaking, explants from first trimester placenta, cultured on collagen I, are often used for study of EVT differentiation and secretory/invasive functions (Knofler and Pollheimer, 2013), while those from later gestation/term placenta, cultured on tissue culture inserts, are generally used to study STB function, including its secretion of various hormones and generation of extracellular vesicles (Fitzgerald et al. 2018; Tong and Chamley, 2018). Dual EVT/STB differentiation has also been achieved from first trimester placental explants, by first stripping of the STB layer to expose the underlying CTB, followed by culture under different oxygen tensions or growth factors to generate either EVT or STB (Caniggia et al. 2000; Baczyk et al. 2006). However, the disadvantage of these models is the presence of a mixed cell type, which makes it difficult to assess or manipulate gene expression in a cell type-specific manner. For this reason, researchers have turned to trophoblast isolation, specifically, isolation of CTB, the trophoblast progenitor cell type.

Using the "Kliman" method (a series of enzymatic digests followed by Percoll gradient centrifugation), or variations thereof, CTB can be isolated which are relatively pure, and can be plated to generate either EVT/STB (from first trimester placenta) or STB alone (from later gestation/term placenta) (Kliman et al. 1986; Fisher et al. 1989). Our own studies have pointed to oxygen tension, through hypoxia-inducible factor (HIF), regulating the switch between STB and EVT differentiation of first trimester CTB cultured on fibronectin (Wakeland et al. 2017). In addition, gene expression can be manipulated in purified CTB, as these cells are susceptible to transduction by lentivirus, at least within a few hours of plating (Chen et al. 2006; Wakeland et al. 2017). However, CTB isolations can be problematic, resulting in low or variable yields and purity, thus requiring additional steps (i.e. using magnetic-activated cell sorting or MACS for further purification) prior to use. Also, since they have a limited life span, at least by traditional culture methods, fresh cell isolates are needed for each new experiment, requiring continuous access to viable placental tissues, which can be difficult, particularly for early gestation tissues. For this reason, researchers have turned to established human trophoblast cell lines, of which multiple have been generated over the years.

Trophoblast cell lines include those generated by immortalization, such as HTR8/SVneo and SW71 cell lines, generated by introduction of either the simian virus large T antigen or hTERT into first trimester trophoblast (Graham et al. 1993; Straszewski-Chavez et al. 2009). Other widely used trophoblast cell lines include BeWo and JEG3 cell lines, derived from gestational choriocarcinoma, a biphasic trophoblastic tumor (Cerneus and van der Ende 1991). However, these cells are suboptimal for studying trophoblast differentiation, as they can only mimic specific phenotypes of either EVT or STB, rather than be stimulated to differentiate from a progenitor to a terminally-differentiated phenotype. In addition, while these cells are easy to culture and manipulate, and provide a much-needed starting point for evaluation of gene regulation and signaling pathways within the trophoblast compartment, they have abnormally amplified and/or aneuploid genomes, and there are significant gaps between their phenotypes and those of bona fide trophoblast cells *in vivo* (Apps et al. 2009; Bilban et al. 2010). For these reasons, and in order to establish a consistent, widely-accessible model of human trophoblast differentiation starting with normal diploid cells, several groups have turned to use of pluripotent stem cells for study of trophoblast differentiation.

Pluripotent stem cells as a model of human trophoblast differentiation

The first derivation of human embryonic stem cell (hESC) lines from outgrowths of preimplantation human blastocysts was reported by Thomson's group in 1998 (Thomson et al. 1998). Shortly thereafter, Xu et al. (2002) reported that hESC can differentiate into hCG-secreting multinucleated cell, resembling placental STB, using feeder conditioned media (FCM) supplemented with bone morphogenetic protein-4 (BMP4). An analogous observation was made when Gerami-Naini et al. reported secretion of hCG, estradiol, and progesterone from hESC-derived embryoid bodies (EBs) grown in suspension then transferred onto Matrigel (Gerami-Naini et al. 2004). Since then, multiple groups, including ours, have confirmed the ability of hESC, and later, that of induced pluripotent stem cells (iPSCs), into trophoblast, along the way making additional observations, further optimizing the protocol, but most importantly, defining the mechanisms underlying this process as it relates to early developmental events in the human embryo.

One of the first mechanistic reports, confirming the involvement of the BMP receptor machinery, was published by Chen et al., showing that hESC lacking phosphatidyl-inositol-glycan class A (PIG-A), required for the first step of glycosyl-phosphatidyl-inositol (GPI) synthesis, failed to induce trophoblast-associated genes, due to the lack of GPI-anchored BMP coreceptors (Chen et al. 2008). At about the same time, another group demonstrated that inhibition of Activin/Nodal signaling in hESC also induced the trophoblast lineage, although BMP signaling was still required for trophoblast differentiation in this context (Wu et al. 2008).

While the initial focus was on the STB lineage derived from these cells, some groups soon began to also note the expression of EVT markers, such as HLA-G, first documented by Das et al. (2007). Others compared BMP4-treated hESC against human mural TE, dissected from human embryos, finding significant overlap in gene expression: one described a "trophectoderm core transcriptional regulatory circuitry" consisting of 13 transcription factors (including GATA2 and GATA3) that was recapitulated by the BMP4-treated hESC model (Bai et al. 2012); another study (Aghajanova et al. 2012) confirmed additional overlap between TE and BMP4-treated hESC in genes coding for TE-secreted proteins, including leukemia inhibitory factor (LIF), PSGs, inhibin, follistatin, and WNT, many of which are known to be involved in embryo implantation (Giudice 1999; Aghajanova 2004). Both concluded, based on the above similarities in gene expression, that BMP4-treated hESC is at least a viable model for studying human trophoblast differentiation.

Our group approached this model, asking the following question: if BMP4 induces terminally differentiated trophoblast lineages, including STB and EVT, does it do so through a developmentally correct program, i.e. through a CTB progenitor phase? We answered this question by probing expression of TP63, a marker of epithelial stem cells which in the human placenta is uniquely expressed in CTB (Lee et al. 2007), in the context of BMP4-treated hESC. We found that TP63 is in fact induced prior to markers of more differentiated trophoblast, and that its knockdown prevented terminal trophoblast differentiation downstream of BMP4 (Li et al. 2013). More recently, Krendl et al. identified steps involved in trophoblast lineage specification of hESC that are upstream of TP63, using a combination of transcriptome and epigenome profiling (Krendl et al. 2017). They identified a combination of four transcription factors (GATA2, GATA3, TFAP2A, and TFAP2C), together called "TEtra," which are expressed in human TE and regulate suppression of pluripotency and induction of trophoblast lineage-specific genes downstream of BMP4 (Krendl et al. 2017).

Despite all the above evidence, the BMP4-based model of human trophoblast differentiation has remained under-utilized, most likely due to under-appreciation of the

differences between mouse and human trophoblast, particularly during early gestation, as well as a lack of understanding of the role of BMP signaling in TE establishment and maintenance. Combined, these issues resulted in the conclusion that BMP4-treated hESC are, at best, extraembryonic mesoderm, not extraembryonic ectoderm (trophoblast) (Bernardo et al. 2011). But, in fact, this study used a suboptimal culture media for trophoblast differentiation of hESC, a media whose basal component was optimized for culture of mouse epiblast stem cells, and not hESC (The International Stem Cell Initiative Consortium, 2010). While the authors correctly pointed out that CDX2 is not trophoblast-specific, and as such, its induction should not be used exclusively to evaluate trophoblast differentiation of pluripotent stem cells, they otherwise relied on mouse placental studies for identification of trophoblast-specific markers. In particular, they pointed to lack of induction of EOMES, a gene whose expression is completely lacking in both pre-implantation human TE and early post-implantation human trophoblast (Blakeley et al. 2015, Soncin et al. 2018), to reject the trophoblast identity of BMP4-treated hESC. Finally, our gene expression profiling of human extraembryonic mesoderm (amnion and placental mesenchyme) and extraembryonic ectoderm/trophoblast (isolated primary CTB) has shown that BMP4-treated hESC cluster more closely with the latter (Li et al. 2013).

With respect to the role of BMP signaling in embryonic patterning, it is true that, prior to the initial report of BMP4-based trophoblast differentiation of hESC (Xu et al. 2002), BMP4 signaling had been mostly studied in the context of mesoderm induction (Zhao 2003). However, there have since been some clues that, in both mouse and human, this pathway is not exclusive to mesoderm induction: specifically, mouse embryos lacking Bmpr1a show not just lack of Brachyury/T (a gene required for early mesoderm differentiation) expression within the embryo-proper, but are also missing Eomes (a gene required for trophoblast stem cell maintenance in the mouse) expression from the extraembryonic ectoderm (Di-Gregorio et al. 2007). More recently, single cell RNAseq of human blastocysts has confirmed expression of multiple components of the TGFβ/BMP signaling pathway in this early stage of development, with several components (including SMAD1 and SMAD5) more highly expressed within human TE (Blakely et al. 2015), indicating that, at the very least, these cells express the machinery required to respond to BMP signaling.

In fact, the balance of mesoderm and trophoblast differentiation downstream of BMP4 treatment of hESC has been documented in several studies. Yu et al. (2011) evaluated the role of FGF signaling, which, in the presence of BMP4, was found to sustain expression of NANOG, leading to induction of BRACHYURY/T, a mesoderm marker. Amita et al. (2013) used this information to establish more optimized conditions for trophoblast differentiation of hESC, applying a combination of BMP4, A83–01 (a TGFβ inhibitor), and PD173074 (an FGF receptor inhibitor). Termed "BAP," this protocol inhibits mesoderm induction and accelerates trophoblast differentiation of hESC, compared to BMP4 alone, rapidly producing terminally-differentiated STB and EVT (Amita et al. 2013). A more recent study has better delineated the mechanism of BMP4-mediated mesoderm vs. trophoblast differentiation. Using both hESC and mouse epiblast stem cells (mEpiSCs), Kurek et al. (2015) showed that BMP4-mediated mesoderm induction, but not trophoblast induction, of these cells is WNT-dependent; thus, inhibitors of WNT signaling can be used to drive differentiation exclusively into the trophoblast lineage. We have used this information to establish an optimized protocol for BMP4-mediated trophoblast differentiation of pluripotent stem cells, using a combination of BMP4 and IWP2 to establish a pure population of CTB progenitor cells (Horii et al. 2019). Note that the need for WNT inhibition is specific to the transition from pluripotency to the trophoblast lineage; this is

distinct from the need for WNT activation in maintenance of the trophoblast stem cell state (see "New models of human trophoblast differentiation: Human trophoblast stem cells" below).

As a model for normal human trophoblast differentiation, pluripotent stem cells (hPSCs) can be useful because they are widely available, and, particularly with the establishment of reprogramming methods for derivation of induced pluripotent stem cells (iPSC) from a variety of human somatic cells (Takahashi et al. 2007), a more widely-acceptable system. Given the pluripotent state of the starting material, this model system allows for the study of, not just human trophoblast differentiation, but also, of human trophoblast lineage specification. In fact, this model system has been used for this purpose, as described above, establishing a role for the "TEtra" factors (GATA2, GATA3, TFAP2A, TFAP2C) in suppression of pluripotency and induction of the trophoblast lineage (Krendl et al. 2017). It has also been used to show that, similar to mouse, the TEA domain protein TEAD4 may be involved in human trophoblast lineage specification, by induction of GATA3 expression, downstream of BMP4 signaling (Home et al. 2012). In our own studies, we have developed a two-step protocol, which separates trophoblast lineage specification from terminal trophoblast differentiation (Horii et al. 2016; Horii et al. 2019). In the first step, we use a combination of BMP4 and IWP2 in a minimal basal media, to derive formation of a pure population of $EGFR^+$ CTB progenitor cells (Horii et al. 2019). These cells are subsequently replated for terminal differentiation (into STB and EVT), using BMP4 in the setting of feeder-conditioned media (FCM) (Horii et al. 2016, Horii et al. 2019). We have used this system to show a possible role for VGLL1, a transcriptional co-factor, in induction of TP63 and human trophoblast lineage specification (Soncin et al. 2018).

But perhaps the most compelling reason to use BMP4-treated hPSCs is for modeling terminal trophoblast differentiation, particularly into EVT, but also into STB, in the setting of both normal development and placenta-based pregnancy disorders, such as recurrent miscarriage, preeclampsia, and fetal growth restriction. Since CTB isolated from term placentae cannot differentiate into EVT (McMaster et al. 1995), the study of this lineage is limited to use of human trophoblast cell lines (i.e. HTR8-SVneo) or CTB isolated from first trimester human placental tissues. Since the latter are primarily sourced from elective terminations of pregnancy, their use may be limited due to both lack of knowledge about their disease potential (unknown pregnancy outcome) as well as local, state, or federal laws in some jurisdictions. We have shown that hPSC-derived CTB can be preferentially differentiated into $HLA\text{-}G^+$, MMP2-secreting, invasive EVT using FCM+BMP4 under low oxygen tension (2%) (Horii et al. 2016). We know that these cells reflect the same process in first trimester placenta, since both CTB derived from these tissues and hPSC-derived CTB differentiate into EVT under low oxygen tension in a HIF-dependent manner (Horii et al. 2016; Wakeland et al. 2017).

Our work has also established proof-of-concept for use of BMP4-treated hPSCs for modeling abnormal trophoblast differentiation. Using hPSCs which harbor an extra chromosome 21, we have shown that STB differentiation is compromised in this setting, similar to STB differentiation of primary CTB derived from Trisomy 21-involved placentae (Horii et al. 2016). Both primary and hPSC-derived CTB with Trisomy 21 showed defects in cell-cell fusion which could be rescued by activin treatment (Horii et al. 2016). Most recently, Sheridan et al (2019) have established iPSCs from a cohort of normal and preeclamptic (PE) pregnancies and, using the "BAP" protocol for trophoblast differentiation of these cells, have shown that PE-iPSC-derived trophoblast display a defect in invasion under high oxygen (20%) tension. Although they have yet to characterize where the defect(s) occur(s) within the PE-iPSC-derived trophoblast, the

fact that the phenotype of a complex pregnancy disorder can be recapitulated using this model system is extremely novel and exciting.

Nevertheless, hPSCs as a model for human trophoblast differentiation have several limitations. Most importantly, even though many human TE- and trophoblast-associated genes are induced in this system, BMP4-treated hPSCs do not fully resemble primary trophoblast based on marker expression (Aghajanova et al. 2012; Li et al. 2013). One glaring difference is the absent-to-low expression of ELF5, a transcription factor required for mouse trophoblast stem cell lineage specification and maintenance, which is also expressed at high levels in trophoblast of the early gestation human placenta (Hemberger et al. 2010). At least in mouse, Elf5 expression is regulated by promoter methylation, with high methylation levels (and hence absent expression) in mouse ESC and low levels (and hence expression of the gene) in mouse TSC (Ng et al. 2008). Human first trimester placental tissues also show hypomethylation of the ELF5 promoter, with ELF5 expressed in villous CTB (Hemberger et al. 2010). Human ESCs, on the other hand, show hypermethylation of the ELF5 promoter, even after treatment with BMP4 (Hemberger et al. 2010), although BMP4 treatment does decrease this methylation (Sarkar et al. 2015). Nevertheless, it is not clear whether, in this setting, this is a limitation of the BMP4-based model, or whether this gene is simply not required for trophoblast lineage specification in human. The latter may be a possibility as, unlike mouse, ELF5 is not expressed in the TE of preimplantation human embryos (Blakeley et al. 2015), with its expression delayed until early post-implantation human trophoblast (Soncin et al. 2018). A second limitation is the continued expression of HLA class I antigens (Bernardo et al. 2011); however, it is noteworthy that the expression of these antigens does decrease following BMP4-induced trophoblast differentiation of pluripotent stem cells (Sarkar et al. 2015). Other limitations of the BMP4-based hPSC model include the as-yet undefined conditions for lineage-specific differentiation into EVT or STB, and the lack of mature markers within either trophoblast subpopulation, indicating that further optimization of terminal trophoblast differentiation is necessary (Yabe et al. 2016; Horii et al. 2019). For this reason, comparison to primary cells continues to be required to improve and validate this system for further use.

New models of human trophoblast differentiation: Human trophoblast stem cells

Over two decades ago, mouse trophoblast stem cells (mTSCs) were first derived from either pre-implantation blastocyst-stage or extraembryonic ectoderm of early post-implantation embryos, using a combination of fibroblast growth factor-4 (FGF4) and media conditioned by mouse embryonic fibroblasts (MEF-CM) (Tanaka et al. 1998). Further studies showed that a combination of FGF4 and TGFβ/Activin signaling contributes to maintenance of these cells in their undifferentiated, stem-like state, and that their removal leads to differentiation into both labyrinthine and spongiotrophoblast/trophoblast giant cell lineages, cells equivalent to human villous and extravillous trophoblast (Tanaka et al. 1998; Erlebacher et al. 2004; Soncin et al. 2015). Since this discovery, mTSCs have been used to study the role of multiple genes and signaling pathways important for trophoblast differentiation and placental development (Rossant 2001; Yagi et al. 2007; Senner and Hemberger. 2010; Home et al. 2017). However, despite many attempts, the same conditions could not be applied to human embryos for derivation of analogous cells, likely due to differences in early developmental stages of mouse and human embryos (Niakan and Eggan. 2013; Kunath et al. 2014; Blakeley et al. 2015). Recently, Okae et al. (2018) applied knowledge of culture conditions for propagation and maintenance of epithelial stem cells to derive human trophoblast stem cells (hTSCs) from both blastocyst-stage human

embryos and early first trimester placentae. These hTSCs use a combination of WNT activation and TGFβ inhibition to self-renew, and can be maintained in culture long-term, and be cryo-preserved for the later usage. They express numerous markers of early gestation trophoblast, including TEAD4, GATA3, TP63, as well as ELF5, and can differentiate into both STB and EVT (Okae et al. 2018).

While the derivation of these cells is a significant advance for the field of placental biology, several questions remain with respect to these cells. First, similar to primary cells from these tissue sources, these cells may remain of limited value due to the ethical and legal challenges against their use and their unknown disease potential. Second, while their profile (including expression of ITGA6) suggests that they originate from CTB, their exact location within the placenta remains unknown. The fact that they could not be derived from later gestation placental tissues may suggest the presence of a specific niche within the first trimester placenta; alternatively, it is possible that TSCs exist in later gestation but in significantly reduced numbers. Several groups have recently analyzed cellular heterogeneity within both the first trimester and term placentae using single-cell RNAseq (Pavlicev et al. 2017; Tsang et al. 2017; Vento-Tormo et al. 2018; Suryawanshi et al. 2018; Liu et al. 2018). Analyses of these data, particularly comparing the CTB cell types at different gestational ages, may shed light on whether the TSC niche is in fact unique to early gestation and determine if this niche can be maintained later into pregnancy. Such a discovery could advance our understanding of placental regeneration, potentially laying the groundwork for targeting of this organ for regenerative therapy.

New models of human trophoblast differentiation: 3D models of the human placenta

While 2D culture is convenient and has been the standard for many years, 3D tissue culture offers conditions that are more physiologically relevant to the *in vivo* environment. Over the past few years, there have been rapid advances in 3D culture systems, with the development of miniature organs or "organoids," as well as establishment of "organs-on-a-chip" (Huh et al. 2011). These advances have recently reached the human placenta field, with multiple groups establishing such model systems.

Specifically, two groups have generated self-replicating trophoblast organoids from first trimester placental tissues (Haider et al. 2018; Turco et al. 2018). Both groups used media with similar composition to that used for hTSCs (Okae et al. 2018), resulting in organoids which hold similar structures, with an outer CTB layer and inner group of STB (Haider et al. 2018; Turco et al. 2018). There were slight differences in passaging and choice of differentiation media. Specifically, Turco et al. chose an EVT differentiation media which is most similar to that used by Okae et al. (2018), containing neuregulin-1 (NRG1) as the initiating factor (Turco et al. 2018); at the same time, Haider et al. showed that removal of WNT activators (R-spondin and CHIR99021) from their organoid media was sufficient to initiate differentiation into $NOTCH1^{+}$ EVT precursors, while WNT signaling was required for further differentiation of EVT (Haider et al. 2018). Similar to hTSCs, trophoblast organoids have only been derived from first trimester placental tissues, again rendering these models of potential limited value due to the ethical and legal challenges to their use and their unknown disease potential, thus necessitating further studies for establishment of similar organoids from later gestation tissues. Another potential limitation of these organoids is that their morphology contrasts with that of chorionic villi, with CTB comprising the outer layer and STB being confined to the inner compartment (Haider et al. 2018; Turco et al. 2018). This results in the presence of a mixed group of trophoblast in culture,

which may confound data analysis; at the same time, the simultaneous presence of different lineages may allow (through cross-talk) better maintenance and/or differentiation of the distinct cell types. In addition, differentiation of the outer CTB into EVT may better recapitulate the trophoblast cell column, allowing the study of progressive differentiation of cells into this lineage (Haider et al. 2018).

Aside from organoids, several other methods of 3D culture have recently been developed to study trophoblast function, mostly in context of the chorionic villus, the main exchange interface of the human placenta. McConkey et al. (2016) used the rotating wall vessel bioreactor to culture JEG3 attached to Cytodex beads, resulting in formation of multinucleated (STB-like) cells which, similar to primary term STB, were resistant to infection by viruses as well as Toxoplasma gondii. More recently, multiple groups have developed "placenta-on-a-chip" models of chorionic villus in order to mimic the transport functions of the placenta *in vitro* (Blundell et al. 2016; Arumugasaamy et al. 2018; Nishiguchi et al. 2019). These models offer an advantage over both 2D models and 3D organoid culture, providing a platform for studying transport of drugs, nutrients, and pathogens across the vasculosyncytial barrier. They can also be modified to model the barrier at different gestational ages and manipulated, both genetically and environmentally, to reproduce disease-like conditions. However, most of these studies so far have used BeWo (choriocarcinoma-derived) cells for modeling the trophoblast barrier, with only one group (Nishiguchi et al. 2019) using primary CTB. Nevertheless, these novel technologies provide a platform for studying transport across the villus barrier, a process which is otherwise difficult to study, relying mostly on ex vivo perfusion of human placenta after delivery. Development of these novel 3D systems will allow the study of cell-cell communications and their effect on placental function significantly advancing our understanding of structure-function relationships within this organ.

Conclusions and Future Perspectives

The human placenta is a difficult organ to study, in part due to lack of a proper model system for its main functional epithelial component, the trophoblast. This review has focused on describing advances in regenerative medicine-based technologies, including derivation of human embryonic and, more recently, trophoblast stem cells, from blastocyst-stage human embryos (**Figure 1A**), derivation of trophoblast stem cells and trophoblast organoids from early gestation human placenta (**Figure 1B**), and derivation of induced pluripotent stem cells and establishment of methods for their differentiation toward the trophoblast lineage (**Figure 1C**). We will now touch on some of the most important possible next steps.

One of the most exciting advances discussed in this review is the ability to reprogram cells of a placenta at delivery, for which the pregnancy outcome is known, generating iPSCs which can then be differentiated into trophoblast (see **Figure 1C**). Currently, this has only been done with mesenchymal stem cells (MSCs) from normal and preeclamptic placentae, with trophoblast differentiation of the resulting iPSCs showing phenotypic abnormalities that correlate with this placental dysfunction (Sheridan et al. 2019). However, much work remains to be done, including more optimal differentiation of iPSCs into trophoblast and establishment of iPSC for modeling other placental disorders. One possibility is to use the media and culture techniques recently developed for hTSC and trophoblast organoids and apply them to iPSC-derived CTB in order to establish iPSC-derived "TSC" (see **Figure 1C**). One group claims to have applied the Okae et al. (2018) media to hESC-derived trophoblast with resulting cells which resemble primary hTSC based on their transcriptome (Mischler et al. 2019). Another more recent

publication first converts hESC/iPSC first into a "naïve" state of pluripotency, then applies the media established by Okae et al. (2018) to derive TSC-like cells (Dong et al. 2020). However, what is not clear is whether this latter method would preserve the disease-specific epigenetic marks on iPSC. Additional studies, particularly comparison of TSC derived from both naïve vs. primed hESC/iPSC, is needed to address this question.

Perhaps a more optimal method for derivation of disease-specific iPSC would be to start by using primary term CTB, instead of MSCs, from diseased placentae, potentially leading to an epigenetic state in the resulting iPSC-derived TSC that is more representative of the cell-of-origin (trophoblast) that we aim to study (see **Figure 1C**). Finally, another exciting possibility would be to apply our knowledge of transcription factors that induce human trophoblast lineage and hTSC maintenance to CTB and/or MSC, and reprogram these cells directly into TSC (i.e. generate "induced trophoblast stem cells" or iTSCs, **Figure 1C**), rather than going through a pluripotent intermediate. This has already been done in mouse, generating iTSC that mimic primary, blastocyst-derived TSC (Kubaczka et al. 2015; Benchetrit et al. 2015).

Just as the Okae media have been applied to hESC/iPSC, so can the culture methods for derivation of trophoblast organoids. Since hESC/iPSC can also be genetically manipulated, the role of specific genes can be tested in both maintenance and differentiation of TSC derived from these cells. In addition, both primary and hESC/iPSC-derived hTSC can theoretically be combined with organ-on-a-chip technologies, in order to model the vasculosyncytial barrier with both normal and abnormal trophoblast. This, combined with the ability to genetically manipulate the component cell types, will allow for modeling of the human placenta, not just during normal development but also in numerous pregnancy-associated disorders, establishing models that can be used for identification of both diagnostic markers and therapeutic targets.

The development of the above technologies has allowed for establishment of significantly more advanced models of human trophoblast. However, much work still remains, particularly in the actual application of these systems for better understanding human placental function and modeling placenta-based pregnancy complications.

Acknowledgement

The authors have no conflict of interest to report.

Author contributions

MH and MMP wrote the manuscript text. OT prepared the first draft of figures. TB assisted with the final version of both the manuscript and figures.

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Figure Legend

Figure 1. A) An illustration of a blastocyst-stage human embryo. Both human embryonic stem cells (ESC) and trophoblast stem cells (TSC) have now been established, derived from the inner cell mass (ICM) and trophectoderm (TE) of human embryos. **B)** An illustration of early gestation (first trimester) human placenta. While primary cytotrophoblast (CTB) derived from this tissue have been the gold standard in studies of human trophoblast differentiation for many years, recent studies have established protocols for derivation of trophoblast stem cells (TSC) and trophoblast organoids, which can self-replicate and be further differentiated into both syncytiotrophoblast and extravillous trophoblast. Utility of both embryo- and first trimester placenta-derived cells is limited by ethical issues as well as their unknown disease potential. **C)** An illustration of human placenta at delivery. Current reprogramming technologies enable us to generate induced pluripotent stem cells (iPSCs) starting with somatic cell types, including placental cells at delivery. This has been done mostly using mesenchymal stem cells (MSCs) derived from the umbilical cord, but can also potentially be done using cytotrophoblast (CTB) as starting material. iPSCs (as well as ESC) can be differentiated into trophoblast using various protocols. Application of TSC/organoid culture media to iPSC/ESC-derived trophoblast has the potential to generate TSC-like cells. At the same time, TSC-specific transcription/reprogramming factors could potentially be used to generate "induced trophoblast stem cells" (iTSC), directly from MSCs or CTBs. This model system is potentially powerful, because it will be applicable not only to normal pregnancies but also to pregnancies associated with placenta-based dysfunction. Note that the blue arrow indicates a method which has already been established and applied, the green arrow represents technology that is available but has yet to be applied, and the red arrow points to protocol(s) that have yet to be developed.

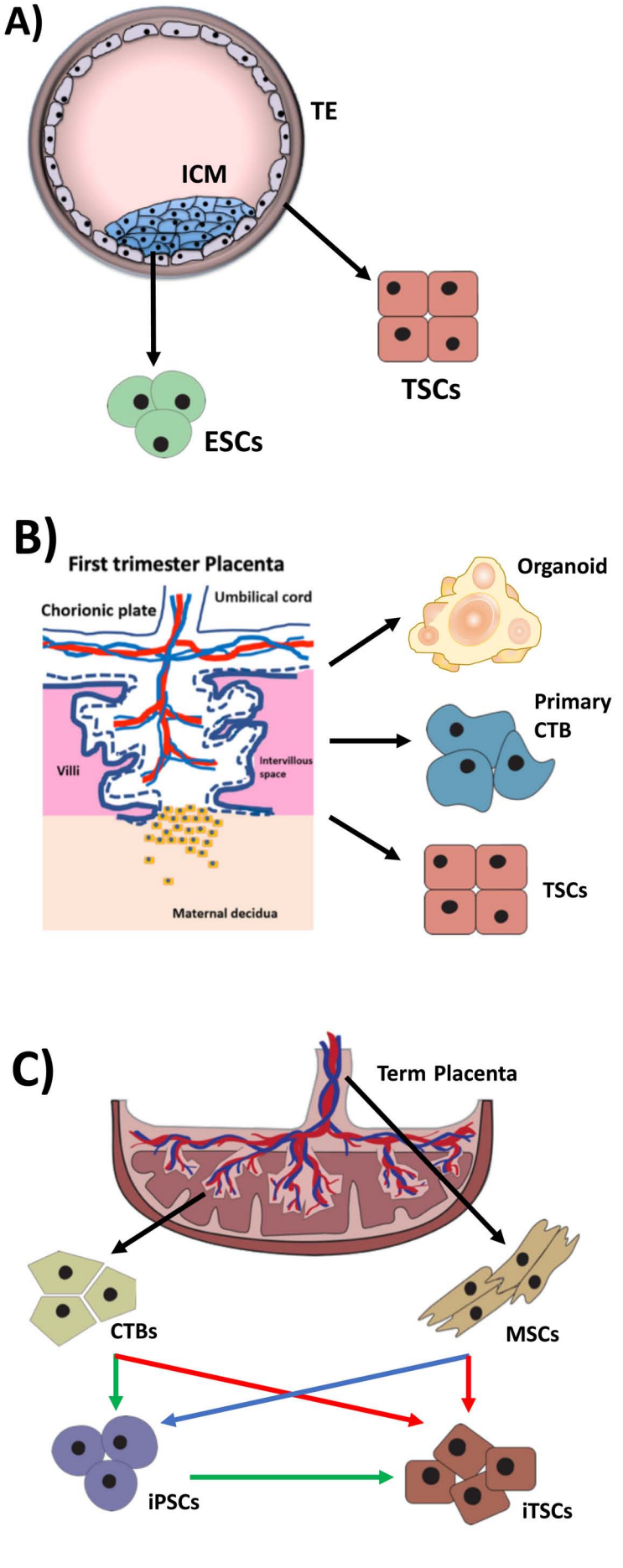
A)
TE
ICM
TSCs
ESCs
B)
First trimester Placenta
Chorionic plate
Umbilical cord
Villi
Intervillous space
Maternal decidua
Organoid
Primary CTB
TSCs
C)
Term Placenta
CTBs
MSCs
iPSCs
iTSCs

Table 1: List of abbreviations

Abbreviation	Definition
BAP	Combination of BMP4, A83–01, and PD173074
BMP4	Bone morphogenetic protein-4
CTB	Cytotrophoblast
EB	Embryoid bodies
EpiSC	Epiblast stem cells
ESC	Embryonic stem cells
EVT	Extravillous trophoblast
FGF4	Fibroblast growth factor-4
GPI	Glycosyl-phosphatidyl-inositol
hCG	Human chorionic gonadotropin
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
hPL	Human placental lactogen
hPSC	Human pluripotent stem cells (includes iPSC and hESC)
ICM	Inner cell mass
iPSC	Induced pluripotent stem cells
iTSC	Induced trophoblast stem cells
LIF	Leukemia inhibitory factor
MACS	Magnetic-activated cell sorting
MEF	Mouse embryonic fibroblast/feeders
MEF-CM	Mouse embryonic fibroblast/feeder-conditioned media
MSC	Mesenchymal stem cells
PSG	Pregnancy-specific glycoprotein
STB	Syncytiotrophoblast
TE	Trophectoderm
TGFβ	Transforming growth factor beta
TSC	Trophoblast stem cells